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(54) Title: METHODS AND MEANS FOR MODULATING THE IMMUNE RESPONSE

(57) Abstract: c-kit (CD117), STAT3, stem cell factor (SCF) and leukaemia inhibiting factor (LIF) are elevated in tolerant immune responses. These may be used in modulation of the immune response generated to an antigen, in particular controlling the presence or degree of tolerance an individual has to an antigen, whether foreign or self. The immune status of an individual, for example with reference to an antigen, may be determined by testing c-kit (CD117), STAT3, stem cell factor (SCF) and/or leukaemia inhibiting factor (LIF) levels.

# METHODS AND MEANS FOR MODULATING THE IMMUNE RESPONSE

The present invention relates to modulation of the immune response generated to an antigen, in particular controlling the presence or degree of tolerance an individual has to an antigen, whether foreign or self. The invention also relates to determining the immune status of an individual, for example with reference to an antigen, in particular whether the individual is tolerant to the antigen, and if so to what degree.

The present invention is based on the finding that c-kit and STAT3 and LIF expression becomes elevated in spleen cells of animals during tolerant responses to an antigen, compared with spleen cells of animals that respond aggressively against the same antigen, as is demonstrated experimentally herein. Determination of c-kit or STAT3 or LIF levels may be used in determination of immune status, whether responding tolerantly or aggressively to an antigen. Also the level of c-kit or STAT3 may be altered, e.g. by provision of additional c-kit or STAT3 polypeptide, e.g. via encoding nucleic acid, or by alteration of endogenous expression levels, or by alteration of polypeptide activity, for example by means of a small molecule or other active agent, in order to modulate the presence or degree of tolerance or aggression that the immune system of an individual shows to an antigen of interest. The present invention in various aspects and embodiments may be used in a variety of contexts, including conditioning of the immune system with respect to a planned transplant, to potential challenge with a pathogen or other foreign body, to transformed cells of the host, e.g. cancer cells or virally-infected cells, and in an autoimmune disorder.

Immune aggression or an aggressive immune response modulated or affected in accordance with the present invention may be an inappropriate immune response, e.g. in an autoimmune disease, or an appropriate immune response, e.g. in response to a pathogen.

Stem cell factor(SCF), sometimes known as "mast cell growth factor", "kit ligand", or "steel factor", is involved in the development of haematopoietic, gonadal, and pigment cell lineages. It has a very wide range of activities with direct effects on myeloid and lymphoid cell development and powerful synergistic effects with other growth factors such as GM-CSF (granulocyte/macrophage colony stimulating factor), IL-7 and erythropoietin.

SCF is the ligand for the c-kit proto-oncogene. Alternative mRNA splicing gives rise to two forms of SCF both of which have a transmembrane domain and are inserted into the cell membrane. The larger form contains a peptide cleavage site and is processed to yield secreted SCF. Both membrane bound and secreted forms are biologically active.

The c-kit proto-oncogene is the receptor for SCF, also known as CD117. C-kit is closely related to the colony-stimulating factor (CSF)-1 receptor and the PDGF receptor. It is expressed on almost all haematopoietic cell precursors, also on mast cells, melanocytes, spermatagonia and oocytes.

LIF (Leukemia Inhibitory Factor) is a pleiotrophic cytokine of 40-45kDa that has a number of roles. See "The Cytokine Facts Book", Academic Press, London 1994 for a review. It is named after its effect on haematopoietic cells and has potent neuromuscular activity, and it has been shown that the transplantation of genetically competent myoblasts into mdx mouse muscle is enhanced when cells are injected with LIF (Kurek, *Clin.*

*Exp. Pharmacol. Physiol.* (2000) 27(7): 553-7). LIF is essential for embryo implantation and associates with Th2 CD4 T helper lymphocytes, and is upregulated by IL-4 and progesterone and down-regulated by Th1 inducers, IL-12, IFN-gamma and IFN-alpha (Piccinni et al. *Biochem. Soc. Trans.* (2000) "8(2): 212-5). A Th2 switch at the level of the materno-fetal interface has been implicated in the maintenance of embryos despite the presence of paternal MHC histocompatibility antigens. Defective production of LIF, M-CSF and Th2-type cytokines by T cells at the fetomaternal interface has also been associated with pregnancy loss (Piccini et al. *J. Reprod. Immunol.* (2001) 52(1-2): 35-43). Bamberger et al. (*J. Clin. Endocrinol. Metab.* (2000) 85(1): 3932-6) reported induction of HLA-G promoter activity by means of LIF administration to JEG3 cells containing a reporter gene under regulation by the HLA-G promoter. HLA-G had been suggested to play a role in facilitating the immune tolerance of the conceptus. Bamberger et al. noted production of LIF in high amounts by the human endometrium and the trophoblast itself, and the presence of LIF receptors on cytotrophoblast cells, suggesting a role for LIF in modulating HLA-G production and immune tolerance at the maternal-fetal interface. LIF has also been shown to enhance signal transduction via STAT3, either directly or indirectly, in the murine model of EAE (Butzkueven et al. Nature Medicine (2002) 8, 613-619).

Signal transduction resultant from SCF binding to c-kit results in autophosphorylation (tyrosine) and tyrosine phosphorylation of mitogen activated phosphokinase-(MAP)-kinase, GAP and PLC-g, as well as Ser Pn of Raf-1. c-kit activation is able to induce signal transduction mediated by the signal transducer and activator of transcription STAT-3. STAT3 is a signal transduction and transcription factor which may be activated by the c-kit receptor. STAT 3 has a role in early embryonic development, in regulation of cell growth, and in organogenesis.

The present inventor has found that levels of c-kit and STAT3 are different in spleen cells which show the properties of specific regulatory tolerance compared with genetically identical spleen cells which show specific immune aggression. Accordingly, the present invention provides for the use of SCF, c-kit, and/or STAT 3, or a substance that affects the activity of any one or more of these, as an agent to regulate the immune response to antigen, whether "foreign" antigen (e.g. allogeneic, xenogeneic, procaryotic, viral or synthetic) or autologous ("self") antigen. This invention also relates to the use of SCF, c-kit, STAT3 and/or LIF in assays of immune status, useful for instance in both clinical and veterinary medicine.

The present invention may be used to guide the immune response of a mammal to accept a transplanted organ, tissue, cell, gene or gene product, artificial substance, or any other agent utilized within the body, e.g. for a therapeutic purpose.

The invention may also be used to guide the immune system to allow for acceptance of, or at least reduced aggressive response to, an antigen associated with an autoimmune disease or disorder, whether eliciting the innate or adaptive immune response during the auto-immune reaction.

The invention may be used to guide the immune response to reject an organ, tissue, cell, pathogen such as a prokaryote, yeast or fungus, parasite or virus, a gene or gene product, an artificial substances, or any other agent that may invade or be taken into the body, or be generated within the body, wherein that agent is unwanted, diseased (e.g. neoplastic tissue or infected tissue), or otherwise deleterious to the host patient.

The invention may be used to enhance the degree of immune response against antigen following vaccination, especially in cases where current vaccination procedures are of limited

success in generating a protective immune rejection response against biological agents, including for example those associated with germ warfare.

In further aspects, the invention provides for enhancing production of autologous or other stem cells or precursor cells and/or immune cells *ex vivo*, e.g for therapeutic purpose. For example, lymphocytes from an individual may be cultured *ex vivo* in the presence of one or more specific differentiation factors (e.g. target antigen for a given TCR) and the response to that antigen adapted, modified or qualified to be regulated for tolerance or to be aggressive to the antigen, using up or down regulation of any one or more of SCF, c-kit and STAT3 activity. The *ex vivo* derived differentiated clones may be propagated and may be used to treat the recipient, especially the original donor, to regulate the immune response. For example, a recipient may be rendered specifically tolerant to a foreign organ allograft prior to receiving the organ graft itself.

Immune response may be guided to tolerance or aggression by signal pathway modulation *in vivo*. On challenge with an antigen, responsive cells may be guided towards tolerance or aggression in accordance with various aspects of the invention. Non-responsive cells, remaining quiescent or dormant, remain unaffected by the regulatory adaptation. The target antigen itself triggers responsive cells or responsive cell populations: cells capable of responding only to other antigens are not triggered, and are therefore not receptive to guiding towards tolerance or aggression towards the relevant antigen at that time. As an alternative or supplement, immune cells may be guided to regulatory tolerance or aggression *ex vivo*. Immune cells of blood and/or spleen may be removed, treated with antigen

and guided to tolerance or aggression, before being returned to the subject.

The present invention in various aspects and embodiments employs c-kit, STAT3 and/or SCF for modulating immune response to an antigen. Unless context requires otherwise, reference herein to "c-kit, STAT3 and/or SCF" should be taken as specific disclosure of each of these components separately and in any possible combination, i.e. c-kit; STAT3; SCF; c-kit and STAT3; c-kit and SCF; STAT3 and SCF; and c-kit, STAT3 and SCF.

As used herein, the term "antigen" refers to any naturally occurring, recombinant or synthetic product such as a polypeptide, which may be glycosylated. The term antigen also includes complexes of protein carriers and non-protein molecules such as steroids, carbohydrates or nucleic acids. Antigen is also used herein to refer to any substance which comprises a plurality of antigens and epitopes, e.g. a cell or tissue, organ, implant, indeed any substance to which an immune response can be mounted by the immune system of a mammal.

The antigen may be an antigen of a pathogenic organism associated with human or animal disease. Organisms which cause animal disease include for example foot and mouth disease virus, Newcastle disease virus, rabies virus and *Salmonella* species. Organisms which cause human disease include for example bacteria such as *Salmonella* species including *S.typhimurium* and *S.typhi*, *Staphylococcus* such as *S.aureus*, *Pertussis*, *Vibrio cholerea*, pathogenic *E.coli*, *Mycobacteria* species such as *M.tuberculosis* and *M.paratuberculosis*. Viral organisms include for example HIV-1 or HIV-2 (which include the viral antigens

gp160/120), HBV (which includes surface or core antigens), HAV, HCV, HPV (e.g. HPV-16), HSV-1 or -2, Epstein Barr virus (EBV), neurotropic virus, adenovirus, cytomegalovirus, polio myelitis virus, and measles virus. Small pox and anthrax are also pathogens of interest and which may be subject to the present invention. Eukaryotic pathogens include yeast, such as *C. albicans*, aspergillus, schistosomes, protozoans, amoeba, plasmodia, including for malaria, toxoplasma, giardia and leishmania.

The antigen may also be a tumour associated antigen. Such antigens include CEA, alpha fetal protein (AFP), neu/HER2, polymorphic endothelia mucin (PEM), N-CAM and Lewis Y.

The antigen may be an abnormally expressed antigen, such as p53 or virally-modified antigen.

Antigens such as those mentioned above may be obtained in the form of proteins purified from cultures of the organism, or more preferably by recombinant production of the desired antigen. Antigens may also be produced by chemical synthesis, for example employing an automated peptide synthesiser such as are commercially available.

"c-kit polypeptide" refers to CD117. A human sequence is available at SWISSPROT P10721; mouse SWISSPROT P05532.

"STAT3 polypeptide" refers to signal transducer and activator of transcription STAT-3. A human sequence is available at GenBank AJ012463, Pietra et al. (1998) Gene 213(1-2): 119-24.

"SCF polypeptide" refers to stem cell factor. A human sequence is available at SWISSPROT P21583; mouse SWISSPROT P20826.

"LIF polypeptide" refers to leukaemia inhibiting factor. A murine sequence is available at SWISSPROT PO9056. A human sequence is available at SWISSPROT P15018.

Instead of wild-type polypeptide, an appropriate fragment may be used provided the desired activity is retained. Similarly, the skilled person is readily able to make changes to amino acid sequence of any polypeptide in a conservative manner, i.e. without abolishing function. Where a nucleic acid sequence encoding c-kit, STAT3 and/or SCF polypeptide is employed, for instance in a gene therapy approach, then changes may be made at the nucleic acid level, and such changes may or may not affect the encoded amino acid sequence, taking into account the degeneracy of the genetic code.

A general aspect of the present invention provides for the use of c-kit, STAT3 and/or SCF for modulating an immune response to an antigen.

One aspect of the present invention provides a method of modulating an immune response to an antigen in an individual, the method including provision in the individual of c-kit, STAT3 and/or SCF polypeptide.

Such provision may be by administration of the polypeptide or polypeptides, or may be by administration of nucleic acid encoding the polypeptide or polypeptides. A further approach comprises administration of a substance that upregulates expression of the polypeptide or polypeptides, e.g. by binding the promoter or other regulatory element of the relevant gene.

The present invention also provides for a method of modulating an immune response of an individual to an antigen, the method comprising administering a substance that affects activity of c-kit, STAT3 and/or SCF in the individual.

The amount of c-kit, STAT3 and/or SCF activity in the individual may be modulated either upwards, so that activity is increased or augmented, or downwards, so that activity is decreased or reduced. Increased activity is associated with a promotion of immune tolerance, while decreased activity is associated with a promotion of immune response against the antigen, i.e. an aggressive response.

Thus, in accordance with the present invention there is provided a method of manipulating the response of the immune system to a given antigen, e.g. increasing tolerance of the immune system of an individual to an antigen, the method comprising administering to the individual c-kit, STAT3 and/or SCF polypeptide, or encoding nucleic acid, or a substance that enhances amount or activity of c-kit, STAT3 and/or SCF polypeptide in the individual.

Further, in accordance with the present invention there is provided a method of potentiating or increasing the aggressive response of the immune system of an individual against an antigen, the method comprising administering to the individual a substance that decreases amount or activity of c-kit, STAT3 and/or SCF polypeptide in the individual.

A substance may decrease activity of c-kit, STAT3 and/or SCF polypeptide by binding or otherwise interacting with it. Such as substance may be for example an antibody molecule with appropriate binding specificity, or other peptidyl or non-peptidyl molecule that binds the c-kit,

STAT3 and/or SCF polypeptide. Amount of c-kit, STAT3 and/or SCF polypeptide (and thus level of activity in an individual) may be decreased by means of a substance that reduces production of the polypeptide, e.g. by down-regulating promoter function of the relevant gene or by targeting encoding mRNA to reduce translation (e.g. by antisense or dsRNA inhibition, RNAi, or ribozyme digestion) or by means of a substance that promotes degradation of the polypeptide, e.g. using ubiquitination.

A substance may increase activity of c-kit, STAT3 and/or SCF polypeptide by means of binding, for instance by binding to c-kit-STAT3 complex enhancing binding between the two component polypeptides, or by binding to a promoter or enhancer region to increase promoter function.

A further aspect of the invention provides a method of enhancing an aggressive immune response against an antigen in an individual, or of providing an enhanced aggressive immune response or reduced aggressive immune response, or of promoting tolerance in an individual, the method comprising administering to the individual a composition comprising the antigen or nucleic acid encoding the antigen and administering a composition which comprises c-kit, STAT3 and/or SCF polypeptide or nucleic acid encoding c-kit, STAT3 and/or SCF polypeptide, or a substance that alters amount or activity of c-kit, STAT3 and/or SCF polypeptide in an individual.

Two or more compositions may be provided as a combined preparation for simultaneous or sequential administration.

A further aspect provides for use of c-kit, STAT3 and/or SCF polypeptide or nucleic acid encoding c-kit, STAT3 and/or SCF polypeptide, or a substance that alters amount or activity of c-kit, STAT3 and/or SCF polypeptide in an

individual as disclosed, in the manufacture of a medicament for administration to a mammal to boost or reduce an aggressive immune response against an antigen or to alter tolerance of the immune system to an antigen, or for use in any method of treatment as set out herein. Such a medicament is generally for administration for treatment or prevention of a disease or disorder associated with the antigen, whether the antigen be of a pathogen, disease cell such as a tumour, or a material to be transplanted, such as an organ, tissue or cell. As discussed elsewhere herein, diseases and disorders that may be treated or prevented in accordance with the present invention include any associated with an antigen of interest and others in which an immune response may play a protective or therapeutic role.

Various aspects of the present invention provide for the use of c-kit, STAT3 and/or SCF polypeptide in screening methods and assays for agents which modulate the immune response to an antigen in a mammal, e.g. increase tolerance to or on the other hand increase an aggressive immune response against the antigen.

Methods of obtaining agents able to modulate an immune response, may determine ability of a test substance to bind with or interact with c-kit, STAT3 and/or SCF polypeptide, and/or ability of c-kit and SCF to bind to one another. A suitable end-point may be used to assess interaction in the presence and absence of a test substance.

In one further aspect, the present invention provides a method for identifying or obtaining an agent which modulates the immune system of a mammal or which modulates the immune response of a mammal to an antigen, the method comprising:

(a) bringing c-kit, STAT3 and/or SCF polypeptide into contact with a test agent; and

(b) determining ability of the test agent to bind c-kit, STAT3 and/or SCF polypeptide.

A test agent found to bind c-kit, STAT3 and/or SCF polypeptide is a candidate modulator, e.g. inhibitor or potentiating agent, of the immune response, e.g. in increasing tolerance of the immune system of a mammal or augmenting aggressive immune response of a mammal against antigen.

An assay method for an agent which modulates immune response of a mammal or tolerance of the mammal to an antigen, may involve determination of binding between c-kit and SCF. Such a method may comprise:

(a) bringing into contact a first substance including a c-kit polypeptide, a second substance including a SCF polypeptide, and a test compound; and,

(b) determining binding between said first and said second substances.

A test compound or agent which reduces or inhibits binding between c-kit and SCF polypeptides may be identified and/or obtained under conditions in which, in the absence of the test compound being an inhibitor, the first and second substances interact.

A test compound or agent which modulates e.g. increases or potentiates interaction between c-kit and SCF may be identified using conditions which, in the absence of a positively-testing agent, prevent or impair the substances interacting. For example, the substance may interact but only weakly, allowing for screening for a substance that enhances binding.

A test compound which increases, potentiates, disrupts, reduces, interferes with or wholly or partially abolishes interaction between said substances (e.g. including a SCF polypeptide and including a c-kit polypeptide), and which may thereby modulate activity of the immune system and may promote tolerance to antigen, may thus be identified.

Another general aspect of the present invention provides an assay method for a substance able to interact with the relevant region of c-kit that binds to SCF, or the relevant region of SCF that binds to c-kit, as the case may be, the method including:

(a) bringing into contact c-kit polypeptide which binds SCF, or a SCF polypeptide which binds c-kit, and a test compound; and

(b) determining binding between said c-kit or SCF polypeptide and the test compound.

A test compound found to interact with the relevant portion of c-kit or SCF may be tested for ability to modulate, e.g. disrupt or interfere with, binding between c-kit and SCF.

Any substance that tests positive in an assay method of the present invention may be tested for ability to affect activity of the immune system of a mammal. In particular, it may be tested for ability to increase or decrease activity of the immune system in an aggressive response to an antigen, and may be tested for ability to promote or inhibit a tolerigenic response.

In any assay method according to the invention, the amount of test substance or compound which may be added to an assay of the invention will normally be determined by trial and error depending upon the type of compound used.

Typically, from about 0.001 nM to 1mM or more concentrations of putative inhibitor compound may be used, for example from 0.01 nM to 100μM, e.g. 0.1 to 50 μM, such as about 10 μM. Greater concentrations may be used when a peptide is the test substance. Even a molecule which has a weak effect may be a useful lead compound for further investigation and development, or as an adjunct to other therapy.

A screening or assay method may further comprise purifying and/or isolating a test compound and/or substance of interest from a mixture or extract, i.e. reducing the content of at least one component of the mixture or extract, e.g. a component with which the test substance is naturally associated. The purifying and/or isolation may employ any method known to those skilled in the art.

The precise format of any of the screening or assay methods of the present invention may be varied by those of skill in the art using routine skill and knowledge. The skilled person is well aware of the need to employ appropriate control experiments.

Compounds which may be screened may be natural or synthetic chemical compounds used in drug screening programmes. Extracts of plants, microbes or other organisms, which contain several characterised or uncharacterised components may also be used.

Combinatorial library technology provides an efficient way of testing a potentially vast number of different substances for ability to modulate an interaction. Such libraries and their use are known in the art, for all manner of natural products, small molecules and peptides,

among others. The use of peptide libraries may be preferred in certain circumstances.

Following identification of a modulator, whether an activator, potentiator or inhibitor of the desired activity, the substance may be purified and/or investigated further and/or manufactured. A modulator may be used to obtain peptidyl or non-peptidyl mimetics, e.g. by methods well known to those skilled in the art and discussed herein. It may be used in a therapeutic context as discussed below.

One class of putative modulator compounds can be derived from c-kit, STAT3 and/or SCF polypeptide sequence. Peptide fragments of these polypeptides or alleles, mutants or derivatives of such fragments may be employed. Nucleic acid encoding such peptides, vectors and host cells containing such nucleic acid, and methods of expressing nucleic acid encoding such peptides are further aspects of the present invention.

Methods of determining binding of c-kit, STAT3 and/or SCF polypeptide to a test substance and of screening for an agent able to modulate the interaction of c-kit and SCF, include methods in which a suitable end-point is used to assess interaction.

Binding may be determined by any number of techniques known in the art, qualitative or quantitative. They include techniques such as radioimmunoassay, co-immunoprecipitation, scintillation proximity assay and ELISA methods. Binding of one substance to a binding partner may be studied by labelling either with a detectable label and bringing it into contact with the other which may have been immobilised on a solid support.

Suitable detectable labels, especially for peptidyl substances include $^{35}S$-methionine which may be incorporated into recombinantly produced peptides and polypeptides. Recombinantly produced peptides and polypeptides may also be expressed as fusion proteins containing an epitope which can be labelled with an antibody.

The polypeptide or peptide which is immobilized on a solid support may be immobilized using an antibody against that polypeptide bound to a solid support or via other technologies which are known *per se*. A preferred *in vitro* interaction may utilise a fusion peptide including glutathione-S-transferase (GST). This may be immobilized on glutathione agarose beads. In an *in vitro* assay format of the type described above a test modulator can be assayed by determining its ability to diminish the amount of labelled peptide which binds to the immobilized GST-fusion peptide (e.g. immobilised fusion peptide of GST and a peptide comprising a c-kit, STAT3 and/or SCF polypeptide). This may be determined by fractionating the glutathione-agarose beads by SDS-polyacrylamide gel electrophoresis. Alternatively, the beads may be rinsed to remove unbound peptide and the amount of peptide which has bound can be determined by counting the amount of label present in, for example, a suitable scintillation counter.

Binding or interaction of polypeptide with a binding partner polypeptide may also be determined using a two-hybrid assay.

For example, one polypeptide may be fused to a DNA binding domain such as that of the yeast transcription factor GAL4. The GAL4 transcription factor includes two functional domains. These domains are the DNA binding domain

(GAL4DBD) and the GAL4 transcriptional activation domain (GAL4TAD). By fusing a polypeptide to one of those domains, and binding partner polypeptide to the respective counterpart, a functional GAL4 transcription factor is restored only when the two polypeptides interact. Thus, interaction may be measured by the use of a reporter gene linked to a GAL4 DNA binding site which is capable of activating transcription of said reporter gene.

This two hybrid assay format is described by Fields and Song, 1989, Nature 340; 245-246. It can be used in both mammalian cells and in yeast. Other combinations of DNA binding domain and transcriptional activation domain are available in the art and may be preferred, such as the LexA DNA binding domain and the VP60 transcriptional activation domain.

Other assays of the present invention for identifying and obtaining substances able to modulate activity of the immune system, include assessing ability of a test compound to affect expression of a c-kit, STAT3 and/or SCF gene, for instance by affecting promoter activity of the gene, either to increase or decrease promoter activity and thus production of the encoded gene product by expression.

A further category of substances able to alter the immune activity of a mammal, especially tolerance, is molecules that affect production of c-kit, STAT3 and/or SCF polypeptide, by means of acting on the promoter of the relevant gene or genes to alter the promoter activity and hence the amount of polypeptide produced by expression from the gene.

By "promoter" is meant a sequence of nucleotides from which transcription may be initiated of DNA operably linked

downstream (i.e. in the 3' direction on the sense strand of double-stranded DNA).

"Operably linked" means joined as part of the same nucleic acid molecule, suitably positioned and oriented for transcription to be initiated from the promoter. DNA operably linked to a promoter is "under transcriptional initiation regulation" of the promoter.

"Promoter activity" is used to refer to ability to initiate transcription. The level of promoter activity is quantifiable for instance by assessment of the amount of mRNA produced by transcription from the promoter or by assessment of the amount of protein product produced by translation of mRNA produced by transcription from the promoter. The amount of a specific mRNA present in an expression system may be determined for example using specific oligonucleotides which are able to hybridise with the mRNA and which are labelled or may be used in a specific amplification reaction such as the polymerase chain reaction. Use of a reporter gene facilitates determination of promoter activity by reference to protein production.

Further provided by the present invention is a nucleic acid construct comprising a promoter region of a c-kit, STAT3 or SCF gene able to promote transcription, operably linked to a heterologous gene, e.g. a coding sequence. A "heterologous" or "exogenous" gene is generally not a modified form of the relevant c-kit, STAT3 or SCF gene. Such a construct may be used in further aspects of the present invention to screen for a substance able to increase or decrease c-kit, STAT3 or SCF polypeptide production in a mammal, and hence to control immune activity, e.g. tolerance of the immune system of the mammal

to an antigen. Generally, the exogenous gene may be transcribed into mRNA which may be translated into a peptide or polypeptide product which may be detected and preferably quantitated following expression. A gene whose encoded product may be assayed following expression is termed a "reporter gene", i.e. a gene which "reports" on promoter activity.

The reporter gene preferably encodes an enzyme which catalyses a reaction which produces a detectable signal, preferably a visually detectable signal, such as a coloured product. Many examples are known, including β-galactosidase and luciferase. β-galactosidase activity may be assayed by production of blue colour on substrate, the assay being by eye or by use of a spectro-photometer to measure absorbance. Fluorescence, for example that produced as a result of luciferase activity, may be quantitated using a spectrophotometer. Radioactive assays may be used, for instance using chloramphenicol acetyltransferase, which may also be used in non-radioactive assays. The presence and/or amount of gene product resulting from expression from the reporter gene may be determined using a molecule able to bind the product, such as an antibody or fragment thereof. The binding molecule may be labelled directly or indirectly using any standard technique.

Those skilled in the art are well aware of a multitude of possible reporter genes and assay techniques which may be used to determine gene activity. Any suitable reporter/assay may be used and it should be appreciated that no particular choice is essential to or a limitation of the present invention.

Nucleic acid constructs comprising a promoter (as disclosed herein) and a heterologous gene (reporter) may be employed in screening for a substance able to modulate activity of the promoter. A substance able to up-regulate or down-regulate expression from the promoter may be sought. A method of screening for ability of a substance to modulate activity of a promoter may comprise contacting an expression system, such as a host cell, containing a nucleic acid construct as herein disclosed with a test or candidate substance and determining expression of the heterologous gene.

The level of expression in the presence of the test substance may be compared with the level of expression in the absence of the test substance. A difference in expression in the presence of the test substance indicates ability of the substance to modulate gene expression. An increase in expression of the heterologous gene compared with expression of another gene not linked to a promoter as disclosed herein indicates specificity of the substance for modulation of the promoter.

A promoter construct may be introduced into a cell line using any technique previously described to produce a stable cell line containing the reporter construct integrated into the genome. The cells may be grown and incubated with test compounds for varying times. The cells may be grown for example in 96-well plates to facilitate the analysis of large numbers of compounds. The cells may then be washed and the reporter gene expression analysed. For some reporters, such as luciferase, the cells will be lysed then analysed.

In a further assay, a STAT3 responsive promoter e.g. operably linked to a reporter gene, is employed in an assay

for alteration of STAT3 function (STAT3 is a transcription factor). The ability of a test agent to affect STAT3 function on a STAT3 responsive promoter may be determined.

Of course, the person skilled in the art will design any appropriate control experiments with which to compare results obtained in test assays.

A further category of substance that may be used to affect the amount of c-kit, STAT3 and/or SCF polypeptide present is RNA, such as antisense, dsRNA, ribozymes and other targetted molecules useful in "gene silencing", that may be used to reduce the amount of c-kit, STAT3 and/or SCF polypeptide produced by expression. The use of anti-sense genes or partial gene sequences to down-regulate gene expression is well established. Double-stranded DNA is placed under the control of a promoter in a "reverse orientation" such that transcription of the "anti-sense" strand of the DNA yields RNA which is complementary to normal mRNA transcribed from the "sense" strand of the target gene. The complementary anti-sense RNA sequence is thought then to bind with mRNA to form a duplex, inhibiting translation of the endogenous mRNA from the target gene into protein. Whether or not this is the actual mode of action is still uncertain. However, it is established fact that the technique works. Antisense RNA oligo- or poly-nucleotides may be administered for take-up into cells to down-regulate expression of the target gene, or may be produced by translation from encoding DNA within cells *in vivo*. The construction of antisense sequences and their use is described in Peyman and Ulman, Chemical Reviews, 90:543-584, (1990), Crooke, Ann. Rev. Pharmacol. Toxicol., 32:329-376, (1992), also Stocks and Rabbitts (2000) EMBO Reports 1(1) 59-64.

dsRNA may be used to downregulate expression. See for example SO01/68836 and WO01/36646.

A still further category of substance able to inhibit polypeptide activity by reducing the amount of polypeptide is ribozymes. These are RNA molecules which can be produced by transcription from encoding nucleic acid, and are able to cut nucleic acid at a specific site - thus also useful in influencing gene expression. Background references for ribozymes include Kashani-Sabet and Scanlon (1995). *Cancer Gene Therapy*, **2**, (3) 213-223, and Mercola and Cohen (1995). *Cancer Gene Therapy* **2**, (1) 47-59.

The present invention accordingly further provides a method of obtaining a modulator of immune activity, e.g. a substance able to promote or inhibit tolerance to an antigen or to promote or inhibit an aggressive immune response against an antigen, the method comprising providing a population of RNA molecules selected from the group consisting of (i) antisense RNA, (ii) dsRNA and (iii) ribozymes, and selecting from the population one or more RNA molecules that are candidate modulators of immune activity by virtue of being able to bind nucleic acid encoding c-kit, STAT3 and/or SCF polypeptide, e.g. the human c-kit, STAT3 and/or SCF gene coding sequence, and/or by virtue of being able to downregulate expression from the c-kit, STAT3 and/or SCF gene. Ability of an RNA molecule to bind a sequence of interest may be determined using any of the suitable techniques available in the art. Ability to downregulate gene expression may be determined by means of quantitation of production of encoded gene product at the polypeptide level and/or by quantitation of mRNA present in the test sample. Degradation of mRNA by a ribozyme results in a lower amount of mRNA being present

and consequently a lower amount of encoded c-kit, STAT3 and/or SCF polypeptide being produced. A ribozyme that degrades c-kit, STAT3 and/or SCF nucleic acid may therefore be used in modulation of an immune system of a mammal by affecting the amount of c-kit, STAT3 and/or SCF polypeptide in e.g. spleen cells of the mammal.

The present invention thus provides in embodiments use of c-kit, STAT3 and/or SCF polypeptide or nucleic acid encoding c-kit, STAT3 and/or SCF polypeptide, in screening for a substance that alters immune activity or immune tolerance to a specific antigen in a mammal, e.g. increase immune aggressive response against an antigen or regulates tolerance, clonal deletion or induction of anergy.

Performance of an assay method according to the present invention may be followed by isolation and/or manufacture and/or use of a compound, substance or molecule which tests positive for ability to bind c-kit, STAT3 and/or SCF polypeptide and/or affect activity of the polypeptide, e.g. by means of modulation of production of the polypeptide by expression from the encoding gene, modulate interaction between c-kit and SCF polypeptides and so on, as disclosed. Following identification of a suitable agent, it may be investigated further, and may be used in modulating or altering immune activity and immune tolerance. Furthermore, it may be manufactured and/or used in preparation, i.e. manufacture or formulation, of a composition such as a medicament, pharmaceutical composition or drug. These may be administered to individuals.

Generally, such a substance according to the present invention is provided in an isolated and/or purified form, i.e. substantially pure. This may include being in a

composition where it represents at least about 90% active ingredient, more preferably at least about 95%, more preferably at least about 98%. Such a composition may, however, include inert carrier materials or other pharmaceutically and physiologically acceptable excipients. As noted below, a composition according to the present invention may include in addition to an modulator compound as disclosed, one or more other molecules of therapeutic use, such as an anti-tumour agent.

Antibody molecules are useful in purification and other manipulation of polypeptides to be employed in aspects and embodiments of the invention and are themselves useful as inhibitors of c-kit, STAT3 and/or SCF polypeptide activity. Other polypeptides, peptides and other substances that bind any one or more of c-kit, STAT3 and/or SCF polypeptide are similarly useful to affect c-kit, STAT3 and/or SCF polypeptide activity.

According to a further aspect of the present invention there is provided a method of obtaining one or more substances that bind c-kit, STAT3 and/or SCF polypeptide and preferably thereby affect immune response or tolerance to antigen in an individual, the method comprising bringing into contact a test substance and c-kit, STAT3 and/or SCF polypeptide, and determining binding of the test substance to the c-kit, STAT3 and/or SCF polypeptide. A test substance that binds c-kit, STAT3 and/or SCF polypeptide may be identified or obtained.

Such a method may comprise bringing a population of antibody molecules into contact with c-kit, STAT3 and/or SCF polypeptide, and selecting one or more antibody molecules of the population able to bind c-kit, STAT3

and/or SCF polypeptide and/or affect c-kit, STAT3 and/or SCF polypeptide activity.

Antibody molecules may routinely be obtained using technologies such as phage display, by-passing direct involvement of an animal's immune system. Instead of or as well as immunising an animal, a method of obtaining antibody molecules as disclosed may involve displaying the population of antibody molecules on the surface of bacteriophage particles, each particle containing nucleic acid encoding the antibody molecule displayed on its surface. Nucleic acid may be taken from a bacteriophage particle displaying an antibody molecule able to bind a peptide or peptides of interest, for manipulation and/or use in production of the encoded antibody molecule or a derivative thereof (e.g. a fusion protein, a molecule including a constant region or other amino acids, and so on). Instead of using bacteriophage for display (as for example in WO92/01047), ribosomes or polysomes may be used, e.g. as disclosed in US-A-5643768, US-A-5658754, WO95/11922.

A peptide or peptides may be administered to a non-human mammal to bring them into contact with a population of antibody molecules produced by the mammal's immune system, then one or more antibody molecules able to bind the peptide or peptides may be taken from the mammal, or cells producing such antibody molecules may be taken from the mammal. The mammal may be sacrificed.

If cells are taken from the mammal, such cells may be used to produce the desired antibody molecules, or descendants or derivative cell lines may be used. Such descendants or derivatives in particular may include hybridoma cells.

Antibody molecules may be provided in isolated form, either individually or in a mixture. A plurality of antibody molecules may be provided in isolated form.

Preferred antibodies according to the invention are isolated, in the sense of being free from contaminants such as antibodies able to bind other polypeptides and/or free of serum components. Monoclonal antibodies are preferred for some purposes, though polyclonal antibodies are within the scope of the present invention.

Antibodies useful in accordance with the present invention may be modified in a number of ways. Indeed the term "antibody molecule" should be construed as covering antibody fragments and derivatives comprising an antibody antigen-binding domain enabling it to bind an antigen or epitope. Example antibody fragments, capable of binding an antigen or other binding partner are the Fab fragment consisting of the VL, VH, Cl and CH1 domains; the Fd fragment consisting of the VH and CH1 domains; the Fv fragment consisting of the VL and VH domains of a single arm of an antibody; the dAb fragment which consists of a VH domain; isolated CDR regions and F(ab')2 fragments, a bivalent fragment including two Fab fragments linked by a disulphide bridge at the hinge region. Single chain Fv fragments are also included.

A polypeptide for use in the present invention, whether c-kit, STAT3 and/or SCF polypeptide, or a peptidyl substance that affects c-kit, STAT3 and/or SCF polypeptide activity or amount e.g. by binding with the c-kit, STAT3 and/or SCF polypeptide (such as an antibody molecule) or by binding with a promoter element that affects c-kit, STAT3 and/or SCF polypeptide production by expression from the encoding gene, or other polypeptide that may be used in any aspect

or embodiment of the present invention, may be produced by recombinant expression.

Nucleic acid is generally provided as DNA or RNA, though may include one or more nucleotide analogues, and may be wholly or partially synthetic. Nucleic acid molecules and vectors according to the present invention may be provided in isolated and/or purified form, e.g. in substantially pure or homogeneous form. The term "isolate" may be used to reflect all these possibilities.

Where it is desired to express a peptide or polypeptide from encoding nucleic acid, the nucleic acid includes appropriate regulatory control sequences. Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids, viral e.g. 'phage, or phagemid, as appropriate. For further details see, for example, Molecular Cloning: a Laboratory Manual: 3rd edition, Sambrook et al., 2001, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in Current Protocols in Molecular Biology, Ausubel et al. eds., John Wiley & Sons, 1992.

Systems for cloning and expression of a polypeptide in a variety of different host cells are well known. Suitable host cells include bacteria, eukaryotic cells such as mammalian and yeast, and baculovirus systems. Mammalian cell lines available in the art for expression of a heterologous polypeptide include Chinese hamster ovary

cells, HeLa cells, baby hamster kidney cells, COS cells and many others. A common, preferred bacterial host is *E. coli*.

A still further aspect provides a method which includes introducing the nucleic acid into a host cell. The introduction, which may (particularly for *in vitro* introduction) be generally referred to without limitation as a transformation, may employ any available technique. For eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection and transduction using retrovirus or other virus, e.g. vaccinia or, for insect cells, baculovirus. For bacterial cells, suitable techniques may include calcium chloride transformation, electroporation and transfection using bacteriophage. As an alternative, direct injection of the nucleic acid could be employed. Marker genes such as antibiotic resistance or sensitivity genes may be used in identifying clones containing nucleic acid of interest, as is well known in the art.

The introduction may be followed by causing or allowing expression from the nucleic acid, e.g. by culturing host cells (which may include cells actually transformed although more likely the cells will be descendants of the transformed cells) under conditions for expression of the gene, so that the encoded peptide or polypeptide is produced. If the peptide or polypeptide is expressed coupled to an appropriate signal leader peptide it may be secreted from the cell into the culture medium. Following production by expression, a peptide or polypeptide may be isolated and/or purified from the host cell and/or culture medium, as the case may be, and subsequently used as desired, e.g. in the formulation of a composition which may

include one or more additional components, such as a pharmaceutical composition which includes one or more pharmaceutically acceptable excipients, vehicles or carriers.

It should be noted additionally that host cells containing nucleic acid encoding a polypeptide useful according to the present invention may be used in therapeutic or prophylactic treatment of an individual, e.g. spleen cells differentiated *ex vivo*, e.g. prior to a transplantation.

A peptide, polypeptide, antibody, nucleic acid or other molecule or agent for use in accordance with the present invention may be formulated into compositions, and are useful in pharmaceutical contexts. These compositions may include, in addition to one of the above substances, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of administration, e.g. oral, intravenous, cutaneous or subcutaneous, nasal, intramuscular, intraperitoneal routes.

Compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous, cutaneous or subcutaneous injection, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

Naked DNA or RNA may be used for expression of an encoded gene product *in vivo*. Naked DNA may be delivered using direct injection or by use of gene-guns (Yang *et al.*, 1990) or any other suitable technique, such as topically e.g. for treatment of psoriasis.

Viral vectors may be used to deliver nucleic acid encoding a desired gene product for production *in vivo*, e.g. a c-kit, STAT3 and/or SCF polypeptide or peptidyl molecule that acts on a c-kit, STAT3 and/or SCF polypeptide or encoding gene to affect amount and/or activity of a c-kit, STAT3 and/or SCF polypeptide *in vivo*.

Nucleic acid encoding a polypeptide or other peptidyl molecule for use according to the present invention may be used in a method of gene therapy. This requires use of suitable regulatory elements for expression and a suitable vector for deliver of the expression unit (coding sequence and regulatory elements) to host cells *in vivo*. A variety of vectors, both viral vectors and plasmid vectors, are known in the art, see e.g. US Patent No. 5,252,479 and WO 93/07282 and countless other publications. In particular, a number of viruses have been used as gene transfer

vectors, including papovaviruses, such as SV40, vaccinia virus, herpes viruses, including HSV and EBV, and retroviruses. Many gene therapy protocols in the prior art have used disabled murine retroviruses. A variety of adenovirus and adeno-associated viral vectors have been developed. Alternatives to viral vectors include transfer mediated by liposomes and direct DNA uptake and receptor-mediated DNA transfer.

A substance to be given to an individual in accordance with an embodiment of the present invention may be administered in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy). A prophylactic effect may be sufficient to potentiate or reduce an aggressive immune response of an individual to a subsequent challenge with antigen (depending on whether an aggressive immune response against antigen or a tolerigenic response is desired). Most preferably the effect is sufficient to prevent the individual from suffering one or more clinical symptoms as a result of subsequent challenge with antigen. A therapeutic effect is sufficient to potentiate or reduce an aggressive immune response of an individual to pre-existing reaction, preferably sufficient to antagonise the reaction, wholly or partially, for example in an autoimmune disorder or in transplant rejection. Most preferably the effect is sufficient to ameliorate one or more clinical symptoms. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery,

the method of administration and other factors known to practitioners. Examples of the techniques and protocols mentioned above can be found in Remington's Pharmaceutical Sciences, 16th edition, Osol, A. (ed), 1980.

A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated and the availability of alternative or additional treatments.

In the present invention, a composition may be administered to an individual, particularly human or other primate. Administration may be to a human or another mammal, e.g. rodent such as mouse, rat or hamster, guinea pig, rabbit, sheep, goat, pig, horse, cow, donkey, dog or cat.

Delivery to a non-human mammal need not be for a therapeutic purpose, but may be for use in an experimental context, for instance in investigation of mechanisms of immune responses to an antigen of interest, e.g. protection against cancers, pathogens and so on.

In further aspects and embodiments, the present invention provides for use of any one or more of SCF, c-Kit and STAT3, and LIF for assaying immune status.

An assay of immune status may be used to assess immune status of an individual in relation to immune response to a pathogen, immune response to a diseased tissue such as a tumour, tolerance to a transplanted tissue, cell or other material (for example to indicate a status of tolerance to an organ allograft or xenograft when it is desired to reduce or remove immunosuppressive therapy to the recipient).

Thus, such an assay may be used in a diagnostic context, to determine the status of the immune system of an individual. It may be used to assess the benefit or success of ongoing treatment.

Generally, embodiments of the present invention may involve an assay of one or more components selected from SCF, c-kit, STAT3 and LIF in an individual to which the assayed component has not or components have not been administered (whether by administration of polypeptide or encoding nucleic acid for production of the polypeptide *in vivo*). For example, in some embodiments, an assay of LIF is performed for an individual to whom LIF has not been provided. In other embodiments, an assay for one or more components selected from the group consisting of SCF, c-kit and STAT3 is performed for an individual to which the component has or components have not been administered.

Assays according to embodiments of the present invention may employ ELISA, Western blot, immunohistochemistry, identification of the effects of drugs on the immune response in terms of induced bias towards regulatory tolerance, anergy or deletion, versus rejection and any other suitable technique available to the ordinary skilled person.

In some embodiments, immune status with respect to aggressive immune response against an antigen or tolerance towards an antigen is determined by means of determination of the presence and amount present of LIF, c-kit, STAT3 and/or SCF polypeptide or encoding mRNA.

There are various methods for determining the presence or absence in a test sample of a particular nucleic acid sequence, e.g. in the context of the present invention mRNA encoding LIF, c-kit, STAT3 and/or SCF polypeptide.

Tests may be carried out on preparations containing cDNA and/or mRNA. RNA is more difficult to manipulate than DNA because of the wide-spread occurrence of RN'ases, which is one reason why cDNA analysis may be performed. Nucleic acid in a test sample may be sequenced and the sequence compared with the known sequence of the LIF, c-kit, STAT3 and/or SCF gene.

However, since it will not generally be time- or labour-efficient to sequence all nucleic acid in a test sample or even the whole gene of interest, a specific amplification reaction such as PCR using one or more pairs of primers may be employed to amplify the region of interest in the nucleic acid if present in the sample. This may be done quantitatively, allowing for determination of the amount of LIF, c-kit, STAT3 and/or SCF mRNA in the test sample.

Nucleic acid may be screened using a specific probe. Such a probe corresponds in sequence to a region of the relevant gene, or its complement Under suitably stringent conditions, specific hybridisation of such a probe to test nucleic acid is indicative of the presence of the nucleic acid molecule of interest, and again this may be quantitated to provide an indication of the amount of such nucleic acid molecule in the test sample.

Specific oligonucleotide primers may similarly be used in PCR to specifically amplify particular sequences if present in a test sample.

A method may include hybridisation of one or more (e.g. two) probes or primers to target nucleic acid. Where the nucleic acid is double-stranded DNA (e.g. cDNA), hybridisation will generally be preceded by denaturation to produce single-stranded DNA. The hybridisation may be as

part of a PCR procedure, or as part of a probing procedure not involving PCR. A screening procedure, chosen from the many available to those skilled in the art, is used to identify successful hybridisation events and may allow for quantitation of the amount of nucleic acid present in the original sample.

Binding of a probe to target nucleic acid (e.g. DNA) may be measured using any of a variety of techniques at the disposal of those skilled in the art. For instance, probes may be radioactively, fluorescently or enzymatically labelled. Probing may employ a standard blotting technique.

A test sample of nucleic acid may be provided for example by extracting nucleic acid from cells such as spleen cells or biological tissues or fluids, urine, saliva, faeces, a buccal swab, biopsy or blood.

There are various methods for determining the presence or absence in a test sample of a particular polypeptide, such as LIF, c-kit, STAT3 and/or SCF polypeptide.

A sample may be tested for the presence of a binding partner for a specific binding member such as an antibody molecule (or mixture of antibodies), specific for the polypeptide or polypeptide of interest. The sample may be tested by being contacted with a specific binding member such as an antibody molecule under appropriate conditions for specific binding, before binding is determined, for instance using a reporter system as discussed. Where a panel of antibodies is used, different reporting labels may be employed for each antibody so that binding of each can be determined.

A specific binding member such as an antibody molecule may be used to isolate and/or purify its binding partner polypeptide from a test sample, to allow for sequence and/or biochemical analysis of the polypeptide to determine whether it has the sequence and/or properties of a LIF, c-kit, STAT3 and/or SCF polypeptide. Amino acid sequence is routine in the art using automated sequencing machines.

A test sample containing one or more polypeptides may be provided for example as a crude or partially purified cell or cell lysate preparation, e.g. using tissues or cells, such as from the spleen or a bodily fluid, preferably blood.

Other tests may involve the use of blood or spleen cells taken from a test animal, individual, subject or patient, and *ex vivo* challenge of the cells with antigen to determine the presence or absence of an aggressive or tolerant response to the antigen.

Spleen cell supernatent may be tested e.g. for LIF, and this may be e.g. by means of ELISA or other method comprising contacting a test sample with anti-LIF antibody and determining the amount or level of binding.

Various other aspects and embodiments will be apparent to the ordinary skilled person in the light of the present disclosure, with reference to the following experimental exemplification.

All documents mentioned anywhere in this specification are incorporated by reference.

*EXPERIMENTAL*

Immune tolerance was generated in a group of mice. An anti-CD4 antibody, YTS 105 (rat IgG2a; Qin et al. (1990) Eur. J. Immunol.

17: 1159) and an anti-CD8 antibody, YTS 177 (rat IgG2a; Qin et al., *supra*), were used according to protocols published by Chen et al. (1992, Eur. J. of Immunol 22: 805, and 1996, Transplantation 62:1200) for the generation of tolerance to a fully mismatched (MHC + mHC) heart graft in mice (e.g. BALB/c graft into CBA mice).

After at least 100 days, BALB/c-tolerant CBA spleen cells from these recipients were used in *ex vivo* studies. For assay of rejection responses, a second group of mice was used, consisting of untreated CBA mice grafted with BALB/c tail skin; this skin was rejected by day 10: BALB/c-rejected spleen cells were removed at 14d for *ex vivo* analyses.

*Ex vivo culture*

Spleen cells from BALB/c-tolerant CBA mice were collected, washed, and resuspended in growth medium (Iscoves MEM plus 10% fetal calf serum). Similarly, spleen cells were prepared from both CBA untreated control mice, and BALB/c-rejected CBA mice. Each set of spleen cells were challenged with irradiated (irr) spleen cells obtained from either CBA, BALB/c, or C57/Bl10 (H-2b), or C57/Bl6 mice. Each culture contained $4x10^7$ responder cells and $6x10^7$ irradiated stimulators in 10ml growth medium. After 5 days, a further $6x10^7$ irradiated stimulator cells were added to re-boost the immune response.

A series of samples was taken at different time points after initial *ex vivo* stimulation and also after boosting at day 5. Samples for analyses included aliquots of growth medium, and cells which were lysed and separated into nuclear and cytoplasmic fractions prior to storage at -80°C in accordance with SD Moffatt PhD Thesis, 1999, University of Cambridge, U.K., Chapter

6, Section II, Part iv, with the following alterations: The cells were resuspended in 200µl BUFFER A lacking NP40 and allowed to swell on ice for 15 minutes, after which 12.5 µl of 10% NP40 solution was added and the mixture vortexed for 10 seconds. The supernatant was spun for 30 seconds at 13,000 rpm, aliquoted and stored at -70°C with no addition of BUFFERC. the nuclear fraction pellet was incubated on ice for 15 minutes after resuspending in BUFFER C, and microfuged for 5 minutes at 13,000 rpm at 4°C. In addition to the 5 day cultures of "self-self" controls, other controls were of freshly isolated CBA spleen cells, and of $2x10^7$ CBA spleen cells cultured for 48h either in growth medium or in supernatant removed from the experimental cultures. All samples for analysis were prepared in an identical manner.

Samples of culture supernatant were also taken at various time points and stored at -80°C until testing by ELISA assay for LIF [R and D Biosystems Cat# MLF00].

*Western blot*

Antibody probes for STAT3, c-kit and SCF were obtained from the company Santa Cruz. Anti-actin was from Chemicon. The Pharmacia Phast ™ system (Amersham Pharmacia Biotech) was used for SDS-PAGE and western blotting of lysates. Western blotting was by standard procedure and probing with ECL-Plus. 1µl of each sample was loaded onto a Pharmacia Phast minigel consisting of 12.5% acrylamide (8 lanes per gel). After running for 54Volthours, the gels were electro-blotted over onto a PVDF membrane, probed for 1 hour with antibody to SCF (sc-9132 in PBS 3%BSA, 2.5% milk, PBS-azide, washed, and reprobed for 30min with horse radish peroxidase (HRP)-linked goat anti-rabbit IgG in PBS 5% milk (dilution 1:20,000). The membrane was rewashed, and probed with tertiary antibody rabbit anti-goat, diluted 1:20,000 in 5% milk-PBS. Using the relevant antibodies, the gel was reprobed after

storage overnight at plus 4°C in PBS-azide, 0.1% Tween™, 5% milk. The first reprobe was for c-kit (sc-5535, 1:200); the second was for STAT3 (sc-9132, 1:350); no tertiary antibody was used for the Stat-3 probe.

In tolerance, it was found that the immune response showed increasing levels of cytoplasmic c-kit which were also detected in the nuclear fraction at 48 hours after primary stimulation; and showed marked stimulation upon reboosting at day 5. By comparison, c-kit in the immune response associated with rejection showed little induction. STAT3 protein levels paralleled those of c-kit, with sustained high expression and only gradual decay with time in tolerance.

In tolerance, culture supernatant at 5 days continued over 300pg/ml LIF, which was more than 10 fold higher than in rejection.

In rejection, the high initial level of STAT3 decayed rapidly: at 3h post boosting on day 5, STAT3 in the rejecting cultures appeared to become degraded into a low molecular weight product of around 80kDa reactive with antibody against STAT3.

The above findings provide indication that, in accordance with the present invention, alteration of SCF, c-kit, STAT3 and/or LIF activity may be used to assay for immune status and to regulate immune tolerance - by increased activity - or to regulate immune aggression - by decreased activity.

CLAIMS:

1. A method for determining immune status of an individual, the method comprising determining level of c-kit (CD117), STAT3, stem cell factor (SCF) and/or leukaemia inhibiting factor (LIF) expression in a test sample comprising tissue, cells and/or bodily fluid removed or obtained from the individual and comparing the level for the test sample with that of a control sample, wherein a level in the test sample greater than that of the control sample is indicative that the immune status in the individual comprises a tolerant immune response, or wherein a level in the test sample lower than that of the control sample is indicative that the immune status in the individual comprises an aggressive immune response.

2. A method according to claim 1 wherein said level is determined for a test sample comprising spleen cells.

3. A method according to claim 1 or claim 2 wherein the control sample is from an individual with an immune status that comprises an aggressive immune response.

4. A method according to any one of claims 1 to 3 wherein the individual has a tissue or cell transplant.

5. A method according to any one of claims 1 to 4 wherein the individual is undergoing therapy.

6. A method according to any one of claims 1 to 5 which comprises determining the level for a component selected from the group consisting of c-kit, STAT3 and SCF.

7. A method according to any one of claims 1 to 5 which comprises determining the level for LIF, wherein LIF has

not been administered to the individual prior to removal of the sample.

8. A method according to any one of claims 1 to 7 wherein level of expression is determined by measurement of the level of c-kit, STAT3, SCF and/or LIF polypeptide in the sample.

9. A method according to any one of claims 1 to 7 wherein level of expression is determined by measurement of the level of mRNA encoding c-kit, STAT3, SCF and/or LIF polypeptide in the sample.

10. Use of determination of expression level of any one or more of the group consisting of SCF, c-Kit and STAT3, and LIF for determining immune status of an individual.

11. Use of c-kit, STAT3 and/or SCF for modulating an immune response to an antigen.

12. Use according to claim 11 wherein the immune response is in an *ex vivo* cell population.

13. Use of c-kit (CD117), STAT3 and/or stem cell factor (SCF) polypeptide in the manufacture of a medicament for treating an individual to enhance a tolerogenic immune response to an antigen in the individual.

14. Use according to claim 13 wherein the individual has an aggressive immune response against the antigen.

15. Use according to claim 13 or claim 14 wherein the medicament is for treating an individual to reduce rejection of transplanted tissue or cells.

16. Use according to any one of claims 13 to 15 wherein the medicament is for treating an individual undergoing other therapy.

17. A method of modulating an immune response to an antigen in an individual or an *ex vivo* cell population, the method including provision in the individual or *ex vivo* cell population of c-kit, (CD117), STAT3 and/or stem cell factor (SCF) polypeptide.

18. A method according to claim 17 comprising provision of the polypeptide or polypeptides by administration to the individual or *ex vivo* cell population of the polypeptide or polypeptides.

19. A method according to claim 17 comprising provision of the polypeptide or polypeptides by administration to the individual or *ex vivo* cell population of nucleic acid which encodes the polypeptide or polypeptides and which expresses the polypeptide or polypeptides in the individual or *ex vivo* cell population.

20. A method according to claim 17 comprising provision of the polypeptide or polypeptides by administration to the individual or *ex vivo* cell population of a substance that upregulates expression of one or more genes encoding the polypeptide or polypeptides.

21. A method of modulating an immune response of an individual or an *ex vivo* cell population to an antigen, the method comprising administering to the individual or *ex vivo* cell population a substance that affects activity of c-kit, (CD117), STAT3 and/or stem cell factor (SCF) polypeptide in the individual or *ex vivo* cell population.

22. A method according to claim 21 wherein the substance increases said activity and thereby increases a tolerant immune response.

23. A method according to claim 21 wherein the substance reduces said activity and thereby increases an aggressive immune response.

24. Use of c-kit, STAT3 and/or SCF polypeptide for screening for an agent which modulates an immune response to an antigen in a mammal.

25. A method for identifying or obtaining an agent which modulates the immune system of a mammal or which modulates the immune response of a mammal to an antigen, the method comprising:

(a) bringing c-kit, STAT3 and/or SCF polypeptide into contact with a test agent;

(b) determining ability of the test agent to bind c-kit, STAT3 and/or SCF polypeptide;

(c) testing a test agent determined to bind c-kit, STAT3 and/or SCF polypeptide for ability to modulate the immune system of a mammal or to modulate the immune response of a mammal to an antigen.

26. A method according to claim 25 wherein step (c) comprises testing with an *ex vivo* immune cell population.

27. A method according to claim 25 wherein step (c) comprises testing *in vivo*.

28. A method according to any one of claims 25 to 27 comprising identifying or obtaining an agent that increases tolerance of the immune system of a mammal to an antigen.

29. A method according to any one of claims 25 to 27 comprising identifying or obtaining an agent that potentiates an aggressive immune response of a mammal to an antigen.

30. An assay method for identifying or obtaining an agent which modulates immune response of a mammal or tolerance of the mammal to an antigen, comprising:

(a) bringing into contact a first substance including a c-kit polypeptide, a second substance including a SCF polypeptide, and a test compound; and,

(b) determining binding between said first and said second substances,

wherein a test compound which reduces or inhibits, or increases or potentiates, binding between c-kit and SCF polypeptides is a candidate for said agent,

(c) testing ability of the candidate to modulate immune response of a mammal or tolerance of the mammal to an antigen, thereby identifying said agent.

31. A method according to claim 30 wherein step (c) comprises testing with an *ex vivo* immune cell population.

32. A method according to claim 30 wherein step (c) comprises testing *in vivo*.

33. A method according to any one of claims 30 to 32 comprising testing the candidate for ability to increase or decrease activity of the immune system in an aggressive response to an antigen.

34. An assay method for identifying or obtaining an agent which modulates the immune system of a mammal or which

modulates the immune response of a mammal to an antigen, comprising:

(a) testing a substance for ability to increase or decrease a c-kit, STAT3 and/or SCF gene expression, whereby a substance found to have said ability is a candidate for said agent, and

(b) testing ability of the candidate to modulate the immune system of a mammal or immune response of the mammal to an antigen, thereby identifying said agent.

35. A method according to claim 34 comprising testing a substance for ability to affect c-kit, STAT3 and/or SCF gene promoter activity.

36. A method according to claim 34 wherein the agent targets c-kit, STAT3 and/or SCF encoding mRNA to reduce translation into polypeptide.

37. A method according to any one of claims 34 to 36 wherein step (b) comprises testing with an *ex vivo* immune cell population.

38. A method according to any one of claims 34 to 36 wherein step (b) comprises testing *in vivo*.

39. A method according to any one of claims 34 to 35 comprising identifying or obtaining an agent that increases tolerance of the immune system of a mammal to an antigen.

40. A method according to any one of claims 33 to 35 comprising identifying or obtaining an agent that potentiates an aggressive immune response of a mammal to an antigen.

41. A method according to any one of claims 24 to 40 further comprising formulating said agent into a composition comprising at least one additional component.

42. A method according to any one of claims 24 to 40 further comprising use of said agent in the manufacture of a medicament for treating an individual or *ex vivo* cell population to modulate immune response to an antigen.

43. A method according to any one of claims 24 to 40 further comprising administering said agent to an individual or an *ex vivo* immune cell population to modulate immune response to an antigen.

44. An agent obtained by a method according to any one of claims 24 to 40.